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Effects of Chronic Mental Stress and atherogenic diet on the immune inflammatory environment in mouse aorta

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Abstract

Inflammation and stress are regarded as two important atherogenic factors. Because stress can affect leukocyte distribution, we hypothesized that stress-mediated leukocyte extravasation can modify the inflammatory environment of the arterial wall possibly contributing to atherogenesis. To test this hypothesis we evaluated the inflammatory environment of the aorta in C57Bl/6 mice subjected to 3 and to 12 months of chronic stress and compared it to age matched non-stressed animals. Experiments were carried out in mice fed regular chow or atherogenic diets. Both treatments increased the expression of vascular and leukocyte adhesion molecules and leukocyte accumulation. At 3 months, stress but not an atherogenic diet elevated the number of CD4 cells, CD8 cells, macrophages, dendritic cells and neutrophils. These changes were associated with elevation of transcripts for ICAM-1 and VCAM-1, E-Selectin and Neuropeptide Y. At 12 months, stress or high cholesterol acted similarly to elevate the number of CD8 and macrophages, and synergistically on the number of all cell types investigated. At this time-point, strong synergism was also observed on the level of E-selectin and NPY in the aorta, but not in the circulation. Despite these effects, histological and morphological alterations of the arterial wall were severe in the atherogenic diet, but not in the stress groups. Thus, although stress and an atherogenic diet may both affect leukocyte accumulation in the aorta, they may contribute differently to atherogenesis.

Keywords

Atherosclerosis; Atherogenesis; Aorta; Chronic Stress; Diet; Inflammation; Adhesion molecules; Neuropeptide Y

Introduction

Atherosclerosis is a chronic inflammatory arterial diseases resulting from the interaction of leukocytes, lipoproteins and cells of the arterial wall (for recent and comprehensive reviews see (Curtiss, 2000; Glass and Witztum, 2001; Hansson, 2005; Libby, 2002). Risk factors for

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its development include smoking, diet, physical inactivity, dyslipidemia, hypercholesterolemia, obesity, hypertension and diabetes mellitus (Thom et al., 2006). In addition, there is epidemiological evidence that psychological stress can contribute to atherosclerosis (Black, 2002; Bosma et al., 1997; Kop, 1997, 1999; Krantz et al., 1996; Lynch, ; Lynch et al., 1998; Muller et al., 1994; Williams and Littman, 1996). In a comprehensive summary of the clinical research in the field, Kop (Kop, 1999) differentiated between three different types of stress (chronic, episodic and acute) and investigated them as risk factors for clinical manifestation of myocardial infarction. Chronic psychological stress, or stress that lasts for over 2 years, was heavily associated with atherosclerosis. Episodic stress, or stress lasting from several months, was associated with altered homeostasis and increased inflammation. Finally, acute stress (i.e. an outburst of anger) was associated with cardiac ischemia, arrhythmia, plaque rupture and thrombosis. This notion found confirmation also in experimental models (Dhabhar and Viswanathan, 2005; Henry et al., 1971; Kumari et al., 2003). Kumari et al. reported that ApoE^{-/-} mice stressed for 12 weeks by mild stressors (restraint and exposure to rat odor) doubled their area of aortic atheromas compared with the non-stressed mice (Kumari et al., 2003). Similar studies were carried out on CBA mice exposed to six months of social confrontation, demonstrating a correlation between chronic social stress and atherosclerosis (Henry et al., 1971). In addition, Li and colleagues (Dhabhar and Viswanathan, 2005) reported that chronic stress induced rapid occlusion of angioplasty-injured rats via Neuropeptide Y (NPY). Factors proposed to mediate the effects of mental stress on atherosclerosis include increased arterial pressure and dysfunctional endothelial responses, reducing bioavailability of NO and enhancing ET-1 formation, therefore affecting vascular flow (Ghiadoni et al., 2000; Luscher et al., 1990).

Stress is also a powerful stimulator of leukocytes redistribution in different species including the mouse and humans (Cohen, 1972; Dale et al., 1975; Dhabhar and McEwen, 1996, 1997; Dhabhar et al., 1994; Fauci, 1976; Fauci and Dale, 1974, 1975; McEwen et al., 1997; Miller et al., 1994; Onsrud and Thorsby, 1981; Spain and Thalhimer, 1951). This phenomenon is regarded as an adaptive response enhancing immune surveillance in compartments more likely to be exposed to infections (i.e. skin) during a situation of danger associated with acute stress. It is primarily mediated by hormones of the hypothalamic pituitary adrenal (HPA) and of the sympathetic adrenal-medullary (SAM) axis that are elevated during stress (for comprehensive review see (Dhabhar, 2007)). Some of these hormones are known to modulate immune functions, including leukocyte trafficking, or to contribute to cardiovascular diseases.

Thus, we hypothesize that stress may contribute to atherogenesis by modifying the immune environment of the arterial wall. To begin testing this hypothesis we performed a characterization of the effects of chronic psychological stress on the immune environment of the mouse aorta. Experiments were carried out in C57Bl/6 mice subjected to chronic stress for up to 12 months. Unlike mice deficient for LDL or ApoE, C57Bl/6 mice are normally non-atherogenic. Yet, data collected with these mice may be more significant with respect to the general human population of individuals that normally do not carry null mutations for lipoproteins. In addition, we intended to test whether prolonged treatment might generate atheromas in wild type mice. Analysis was performed in animals maintained on normal chow diet or on atherogenic diet rich in cholesterol. Leukocyte composition and distribution as well as the levels of adhesion molecules and of other pro-atherogenic markers were assessed.

Methods

Mice and stress treatments

All procedures were approved by the AIACUC, with standards of the American Association for the Accreditation of Laboratory Animal Care (AAALAC) and the regulations set forth in the Animal Welfare Act. Experiments were initiated in 3 month old C57Bl/6 male mice (n=8/group) housed 4 per cage under a 12:12 hrs dark:light cycle with light on at 6 am; food and water were provided *ad libitum*. Stress experiments were carried out between 9AM and 6PM. Mice were left undisturbed or were subject to the following stressors: *Restraint stress*: mice were placed in individual vented plexiglass restrainers that prevented them from inverting their position for 6 hrs (Barr and Phillips, 1998; Conti et al., 1997; Conti et al., 2000; Michel et al., 2005); *Conspecific exposure*: two animals (each from a different cage) were placed together in a new cage for dominance establishment (Barr and Phillips, 1998; Michel et al., 2005). To ensure stress, the animals were placed in a new cage with clean bedding devoid of signals (i.e. urine scent) of previously established dominance. 3. *Water Avoidance*: a clean empty cage was filled with 0.3 cm of water; a 1 cm high 3.0 cm wide platform was provided as a dry environment (Levine, 2000). *Damp bedding*: water sufficient to wet the bedding without generating pools was added, where mice stayed for 6 h (Barr and Phillips, 1998; Michel et al., 2005). To avoid habituation, stressors were assigned in random order while assuring that at the termination of the experiments each animal received the same number of stressors. Treatments were carried out with daily stress sessions of 6 hrs for 5 consecutive days followed by 2 days of rest.

Diet

Diets were purchased from Harlan. Regular chow (ND, Normal diet, Breeder diet): S2335 7004: 4% saturated, 4.6% mono-unsaturated and 2.4% polyunsaturated fat, and 45% carbohydrates, totalizing 11.7% of kcal from fat. Atherogenic diet (HC, high cholesterol, Western diet): Teklad, TD.02028: 1.25% Cholesterol. & 0.5% Cholic Acid, 13.78% saturated, 6.57% mono-unsaturated and 0.85% polyunsaturated fat, and 41% carbohydrates, totalizing 42.6% of kcal from fat).

Aorta Dissection

At termination time-points, blood was harvested for serum and animals were anesthetized with isoflurane (4–5%) and intracardially perfused either with phosphate-buffered saline (PBS; pH 7.2) or with a solution of 4% paraformaldehyde in PBS. Under a dissection microscope, whole aortas were collected, freed of adipose tissue, and processed according to the procedure that followed. For flow cytometry, whole aortas were digested as described (Galkina et al., 2006) with 125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I-s, 60 U/ml DNase I, and 450 U/ml collagenase type I (all enzymes were obtained from Sigma-Aldrich) in PBS/20 mM Hepes at 37°C for 1 h. Cell suspensions were obtained by straining the aorta through a 70-µm mesh. To optimize the number of animals employed, aortic arches were used for transcriptional analysis, and part of the trunk and bifurcations were preserved in Carnoy's fixative for 48hs, followed by 70% ethanol for paraffin-embedding for histology.

Flow Cytometry

Cells were incubated with Abs for 20 min at 4°C and washed twice. Fluorescence was detected by flow cytometry (FACSCalibur, BD Biosciences), and data was analyzed using FlowJo (Tree Star Inc.) software. The antibodies used were PeCy5-CD45RA, FITC-CD3, APC- CD8, PerCP- B220, PE-Mac-1, PeCy5-Gr1 and APC-CD11c (all from BD Biosciences). Leukocytes retrieved from Normal diet Controls (NDC), Normal Diet Stress

(NDS), High-Cholesterol Control (HCC) and High-Cholesterol Stress (HCS) groups were characterized as follows: lymphocytes were CD3+CD4+ or CD3+CD8+; macrophages were CD3- CD11b+ CD11c- Gr1low; myeloid Dendritic Cells were CD11b+ CD11c+ and neutrophils were CD11b+ Gr1 high cells.

Molecular profiling using qRT-PCR

RNA was extracted from aorta arches and trunks separately by using a Fibrous Tissue RNA Extraction kit (Qiagen) and retro transcribed by RT2 First Strand cDNA kit (Qiagen). Genomic DNA contamination was eliminated by Dnase treatment by using Rneasy Micro Kit (Qiagen). Mouse Atherosclerosis RT2 Profiler PCR Array and RT2 Real-Timer SyBR Green/ROX PCR Mix were purchased from SABiosciences/Qiagen (Frederick, MD). PCR was performed on ABI Prism 7900HT Fast Sequence Detector (Applied Biosystems). For data analysis the ddCt method was used; for each gene fold-changes were calculated as difference in gene expression between controls and stressed animals.

ELISAS and EIAs

Corticosterone was measured using an EIA kit exhibiting a 30 pg/ml detection limit, 80% accuracy, intra-assay precision of 8–13% and inter-assay precision of 3–17% (Cayman Chemical, Ann Arbor, MI), following the manufacturer's instructions. Soluble E-Selectin was measured in the 50-fold diluted serum using the Mouse E-Selectin Immunoassay with a detection limit of 2.8 pg/ml, 89–97% accuracy, both intra-assay and inter-assay precision of 6–8 % (R&D Systems, Minneapolis, MN), following the manufacturer's instructions. Neuropeptide Y (NPY) was also measured in the serum using Rat/Mouse NPY ELISA kit with a detection limit of 2 pg/ml, 89–93% accuracy, intra-assay precision of 1–3% and inter-assay precision of 6–12% (Millipore Corporation, Billerica, MA), following the manufacturer's instructions. Samples were assayed in duplicate and plotted against standard curves provided by the kits for determination of concentrations in the serum.

Immunohistochemistry

Cells—The primary antibodies used were CD68 (Serotec) for macrophages, CD11c (BD Biosciences) for dendritic cells, and CD31 (BD Biosciences) for endothelial cells. Secondary antibodies were goat anti-mouse and -rat IgG conjugated to Alexa Fluor 488, 568, or 647 (Invitrogen). Aorta segments of 3 mm² were stained in 0.1-ml droplets of staining buffer containing dilutions of previously titrated primary antibodies. Segments were initially incubated in 10% normal goat serum (Vector) for 60 min at room temperature. Tissue was permeabilized with 0.2% Triton X-100 and 0.1 M-glycine in PBS for 7 min at room temperature. Immunofluorescence antibody staining was performed in a stepwise manner, with each step accomplished by overnight incubation in the dark at 4°C on a rotating mixer. Between staining steps, segments were washed in PBS containing 0.1% Tween-20. The cut open segments were placed on a glass slide, mounted, and pressed overnight. Sample visualization was performed with a confocal microscope (2100 Radiance; Bio-Rad Laboratories). The 60× and 100× objectives were used to collect high-resolution images with an x–y resolution of 0.15 μm and a z-distance resolution of 0.80 μm. To characterize cells within the aorta intimal layer, z-series optical sectioning was performed with the 60× or 100× objectives, creating image stacks that span the thickness of the intimal layer. Z-series image stacks were collected at 0.5 μm z-distance increments. In the z-series images, green autofluorescence and background, were sorted from bright staining. The 20× objective was used to create composite images, generated by stitching contiguous images that spanned the lesser curvature either vertically (circumferential strip) or horizontally using Photostitch (LEAD Technologies). Three-dimensional analysis of regions of interest was performed on ImageJ (National Institutes of Health).

NPY and E-Selectin—Aorta bifurcations were collected and fixed in Carnoy's fixative for paraffin embedding. IHC staining followed a basic indirect protocol using a citrate antigen retrieval method on 6 μ m sections, using anti-NPY (rabbit polyclonal, Bachem, Torrance, CA) and E-Selectin (SELE rabbit polyclonal antibody, Novus Biologicals, Littleton, CO), as previously described (Roberts et al., 2003). Images were visualized and acquired using a Zeiss (Oberkochen, Germany) Axiovert 200 inverted microscope at 20 $\times$ or 100 $\times$ magnification and captured by using the Zeiss AxioCam HRC associated with Zeiss Axiovision 2.0.5 software package. Negative controls were performed by omitting primary antibodies.

Statistical analysis

Values in figures represent Mean $\pm$ SD of 5–8 animals per group, as indicated in the Figure legends. Tests were performed using Excel (Microsoft Corporation, Redmond, WA) and Prism software (GraphPad Software, San Diego, CA) for Macintosh. For the comparison of 4 conditions, One or Two-Way ANOVA was followed by Bonferroni's post-hoc test. Lines delimit groups in which significance was observed. * indicates $p < 0.05$.

Results

Stress and diet

Prior to the initiation of stress regimens, the efficacy of each individual stressor was monitored in single sessions by evaluating the circulating level of corticosterone. Compared to control (133.8 ± 16.57 ng/ml), all four stressors induced robust corticosterone elevation: restraint stress, 348.38 ± 31.98 ng/ml; water avoidance, 353.5 ± 61.75 ng/ml; conspecific exposure, 325.63 ± 15.83 ; damp bedding, 377.27 ± 9.35 ng/ml). The efficacy of stress over time was monitored by evaluating weight gain at the beginning and at the end of each 5-day stress period. Over time, stressed animals fed chow (ND) showed on average 17.1% (± 3.74) lower body weight than the unstressed controls, whereas stressed animals fed an atherogenic high cholesterol (HC) diet showed 9.6% (± 1.46) lower body weight than unstressed HC mice. Food intake was also evaluated to investigate whether preference or amount of calories consumed per day could influence the outcome of the experiments. During the first 15 days of diet, animals fed ND or HC diet consumed a similar amount of calories (12kCal/day). Subsequently, animals receiving HC diet increased their consumption, which reached a stable 15–18 kCal/day, while ND animals maintained a 12–14kCal consumption/day. The introduction of stress elevated the consumption of food in kcal by 16.4% (± 2.1) in ND and 11.3% (± 2.6) in HC. The time length of stress exposure was also taken into account.

Leukocyte numbers

The numbers of total leukocytes, CD4 and CD8 lymphocytes, dendritic cells (DC), macrophages and neutrophils were measured from whole aorta cell suspensions using flow cytometry (FACS). Measurements were carried out in the following four groups: normal diet controls (NDC), normal diet stressed (NDS), high cholesterol atherogenic diet controls (HCC) and high cholesterol atherogenic diet stressed (HCS). Stress was applied for a period of 2 weeks, 3 months or 12 months.

Two weeks—Two weeks of stress and/or atherogenic diet did not cause significant changes in any of the cell types investigated compared to control non stressed and/or normal diet groups (not shown).

Three months—At 3 months of treatment, elevation of leukocyte numbers was observed across all groups. NDC mice had an average of 11.9×10^3 (± 3.1) total cells per whole aorta retrieved from the whole digested aortae, 55% of them were CD45RA+ blood leukocytes

(Charbonneau et al., 1988). Within the CD45RA+ fraction, we identified $0.74 \times 10^3 (\pm 0.22)$ CD4+ T cells, $3.1 \times 10^3 (\pm 1.4)$ CD8+ T cells, $2.0 \times 10^3 (\pm 0.87)$ CD11b+ CD11c- Gr1low macrophages and $0.27 \times 10^3 (\pm 0.12)$ CD11b+ CD11c+ dendritic cells (DCs). The number of B220+ cells approached background in all animals. Compared to NDC, leukocyte accumulation increased by 3.4-fold following stress and by 2.6-fold on atherogenic diet. The effects of stress were observed also on high cholesterol diet where the total number of leukocytes increased by 1.39-fold compared to HCC. Stress elevated all cell types analyzed in both ND and HC groups. Atherogenic diet elevated the number of CD4 and CD8 T lymphocytes but had no significant effects on macrophages, dendritic cells and neutrophils (Figure 1A). Compared to NDC, CD4 cells increased by 5.2-fold in NDS mice, by 2.8-fold in HCC and by 6.8-fold in HCS suggesting an additive effect of stress and atherogenic diet. Stress showed the strongest effects on CD8 cells: compared to NDS the number of CD8 lymphocytes increased by 11.2-fold in NDS and 7.8-fold in HCS. Atherogenic diet also elevated the number of CD8 cells (4-fold in HCC compared to NDC) but did not have additive effects with stress. Finally, stress significantly increased macrophage, dendritic cell and neutrophil accumulation similarly (by 3.5–4-fold) in both normal and high cholesterol groups.

Twelve months—The total number of cells isolated from NDC aorta cell suspensions was $39.2 \times 10^3 (\pm 16.2)$, 67% of them were CD45RA+ leukocytes, of which $4.1 \times 10^3 (\pm 2.0)$ CD4+ T cells, $9.8 \times 10^3 (\pm 3.9)$ CD8+ T cells, $9.6 \times 10^3 (\pm 3.0)$ CD11b+ CD11c- Gr1low macrophages and $0.47 \times 10^3 (\pm 0.21)$ CD11b+ CD11c+ dendritic cells (DCs). In animals subjected to 12 months of treatment the values for total leukocytes increased by 3-fold in the stress group and 2-fold in the high cholesterol group, and 4.8-fold in the group receiving both treatments combined. Although the increment was similar to what was found at 3 months, important differences were observed when analyzing individual cell subpopulations (Figure 1C). Chronic stress combined with either normal diet or high cholesterol diet increased leukocyte subset numbers similarly and with a magnitude comparable to that observed at 3 months the number of macrophages (~2-fold), CD8 (~5-fold) and dendritic cells (~2–3-fold). However, unlike at 3 months, stress or atherogenic diet alone had no effects on CD4 cells and neutrophils. Instead, the combined action of stress and atherogenic diet strongly elevated the level of all cells investigated. These effects were additive for CD8 (~11-fold) and dendritic cells (~5-fold) and were synergistic for CD4 cells (~11-fold). Macrophages (~16-fold) and neutrophils (~14-fold) were otherwise not affected by single treatments.

Transcriptional analysis

We analyzed the expression of multiple transcripts known to be relevant in the development of atherosclerosis. Table 1 shows ddCT relative values for all transcripts analyzed by qRT-PCR in all experimental groups, at 3 and 12 months of treatment.

Three months of stress robustly increased the expression levels of the adhesion molecules: intracellular adhesion molecule-1 (ICAM-1, CD54), vascular adhesion molecule-1 (VCAM-1, CD106) and E-Selectin (CD62E). The effects were similar for mice on normal or on atherogenic diets (ICAM-1, 143 ± 94.6 -fold on ND, 66.5 ± 49.6 -fold on HC; VCAM 2004 ± 713 -fold on ND, 3731 ± 874 -fold on HC; E-Selectin, 427 ± 83 -fold on ND, 197 ± 88 -fold on HC). Stress also elevated the level of the transcripts for fatty acid-binding proteins (Fabp) (15.2 ± 3.78 -fold on ND, 6.11 ± 2.7 -fold on HC), for adipophilin (Adfp) (13.5 ± 7.72 -fold on ND, 10.11 ± 4.7 -fold on HC) and for neuropeptide Y (NPY) (8.22 ± 2.46 -fold on ND, 28.8 ± 4.4 -fold on HC). The increase in the expression of these molecules correlated with the profile of leukocytes enrichment following stress. None of these markers were significantly affected by high cholesterol diet (Figure 1B), which instead affected the

expression of Von Willebrand Factor (Vwf - 14-fold in comparison to controls under ND) (Table 1).

In animals that were stressed for 12 months, the transcripts of ICAM-1 and VCAM-1 were also increased to above control levels although to approximately 28- and 30- fold lower, respectively, than after 3 months of treatment. A similar elevation was observed not only in stressed animals but also in HC-fed mice (Figure 1D). In contrast, E-Selectin (CD62E), was not affected by either stress or cholesterol alone but was elevated 3620-fold by their combined action. A similar pattern was observed for Neuropeptide Y (NPY), which was increased 2465-fold by both factors together. Fabp3 was elevated from 3–9-fold only in animals that were fed high cholesterol diet, regardless of stress. Levels of Adpf transcripts were instead elevated equally by both treatments alone (11–19-fold) and that synergistically (109-fold) by their combined action.

Histology and atherosclerotic plaque evaluation

Histological analysis performed on the bifurcation of the aorta, towards the femoral artery (Figure 2 – upper panels) showed that although flow cytometry and transcriptional analysis suggested significant changes caused by stress at 3 months of treatment, the morphology of the aorta tissue not detectably affected by either stress or HC. In contrast, in animals subjected to treatments for 12 months, high cholesterol and stress, individually or together, distinctive morphological changes were evident, which were increasingly severe (Figure 2 – lower panels). High cholesterol alone caused a significant loss of architectural integrity, with the development of a lining of fat cells, which stained positively for Mac-3 (not shown), suggesting that long term HC diet leads to foamy cells in WT tissue. Surprisingly, though, animals receiving HC and stress for 12 months did not show morphological changes related to foamy cell development, but a higher cellularity that was partially due to the accumulated leukocytes (Figure 2). For the analysis of localization of accumulating cell types we have focused on macrophages and DCs, at the lesser curvature of the aortic arch. The enrichment of macrophages in the intima of animals receiving HC diet and stressed for 12 months was observed in the aorta tissue using a CD68 label, in association with CD31-labelled endothelial cells (Figure 3A). CD11c+ DCs were also observed in the intima of 12-month treated animals, but these cells were not capable of penetrating towards the adventitia, localized at 200µm of the z-axis (Figure 4). The examination of the adventitia by immunostaining did not show accumulation of these two cell types. The three-dimensional analysis reveals that the macrophages and DCs are predominantly located at the intima (0 value on z axis), with some penetration towards the adventitia (200 µm on z axis) (Figure 4). We have not investigated the localization of lymphocytes and neutrophils using this technique.

The upregulation of NpY and E-Selectin in animals subjected to both insults for 12 months was further confirmed by immunohistochemistry (Figures 3B). NpY was lightly expressed by endothelial cells and platelets, and highly expressed in leukocytes. Another interesting observation was the presence of platelet aggregates in HCS (Figure 5B). The platelet aggregation observed in the vessels of HCS was not correlated with an exclusive elevation of P-Selectins, since this molecule was elevated in both HC groups equally at that time point (Figure 5A).

Evaluation of atherosclerotic plaque was carried out on entire aortas. No plaques were observed in any of the experimental groups and conditions tested (not shown).

Soluble Biomarkers

Given the remarkable synergistic effect of stress and atherogenic diet on the level of E-selectin and NPY transcripts, we measured plasma levels of soluble isoforms of these two molecules to determine if they may represent markers of stress and diet-induced atherogenesis (Figure 6). The levels of circulating sE-selectin were similar in all experimental groups at 3 and 12 months. A modest but statistically significant increase was found only in 3 months HCS compared to age matched NDS (49.53 ± 1.12 ng/ml in HCS and 41.40 ± 3.01 ng/ml in NDS, $p=0.046$) (Figure 6A). Circulating NPY increased significantly only in atherogenic groups when comparing 12 month treated groups (0.514 ± 0.13 ng/ml in HCS compared to 0.18 ± 0.04 ng/ml in NDS, $p=0.002$, or compared to 0.31 ± 0.02 , $p=0.041$) and showed the opposite trend in 3 month treated groups although without reaching statistical significance (Figure 6B).

Discussion

We evaluated the effects of prolonged chronic stress on the number and types of the main leukocytes subsets known to participate to atherosclerosis (Galkina and Ley, 2009). The stress models employed utilized four different stressors that can be considered primarily psychological/mental rather than physical. Mice were fed regular chow diet or an atherogenic diet rich in cholesterol to investigate the effects of the interaction of these two risk factors. Treatments were performed for different lengths of time, up to a maximum of 12 months, corresponding approximately to one third of the length of a mouse lifespan. Habituation was avoided by utilizing random assignment of stressors and was demonstrated by monitoring body weight gain.

We chose to use the C57Bl/6 wild type mice to evaluate whether prolonged chronic stress or its combination with atherogenic diet may induce the formation of plaques in this otherwise non- atherogenic prone strain. In addition, the results obtained with these animals may be more significant to human health as the majority of individuals do not have lipoprotein deficiencies required to investigate plaque development in mice. In addition, in transgenic models of atherosclerosis such as ApoE^{-/-} or LDL receptor-deficient mice, the analysis of subtle events can be potentially masked by the severity of the pathology, which leads to lesions in a very short time. We did not observe development of atherosclerotic plaque in the groups investigated indicating that neither stress alone nor its combination with atherogenic diet were effective in inducing atheromas in C57Bl/6 mice.

Profound changes were observed in the immune environment of the aorta. Three months of stress elevated the number of CD4 and CD8 cells, macrophages, dendritic cells and neutrophils while high cholesterol diet increased only CD4 and CD8 cells and macrophages. In addition, stress induced more cell accumulation than high cholesterol suggesting that stress may be a more powerful modulator than atherogenic diet on aorta leukocyte accumulation. Differences in the kinetics of adhesion ligands on leukocyte subsets by either risk factor were not investigated, but could also potentially explain the selective effect of high cholesterol in comparison to stress, and the higher degree to which stress induced cell adhesion at early time-points. Analysis of the aorta transcriptional profile showed that ICAM-1, VCAM-1, E-Selectin and P-Selectin correlated strikingly with the effects of stress. VCAM-1, E-Selectin and P-Selectin are predominantly expressed in endothelial cells suggesting that their increase was mostly attributed to changes in these cell types. Instead, ICAM-1 is also expressed in leukocytes. All three adhesion molecules were previously demonstrated to be instrumental in the development of atherosclerosis and allow the interaction between leukocytes and epithelial cells, suggesting they may mediate the increase of aorta leukocytes (Cybulsky et al., 2001), (Dansky et al., 2001; Hulthe et al., 2006), (Roldan et al., 2003; Sobey, 2003) (Bourdillon et al., 2000; Collins et al., 2000). The

atherogenic diet, while still inducing elevation in aorta lymphocytes and macrophages, it did not alter the level of the adhesion molecules investigated. It is thus possible that stress and high cholesterol diet affected the number of CD4, CD8 and macrophages in the aorta by different means, including the use of different adhesion molecules. Another interesting possibility, not investigated in this study, is that these risk factors differentially affected proliferation of resident cells as opposed, or in addition to adhesion of circulating cells.

In addition to adhesion molecules three other transcripts correlated with the profile of aorta leukocytes at 3 months: that encoding for neuropeptide Y (NPY), and those for the fatty acid-binding proteins 3 (Fabp3) and adipophilin (Adfp), both involved in lipid metabolism. Neuropeptide Y (NPY) is a neurotransmitter found in both the central and the peripheral autonomic nervous system, including the adventitial nerves, but is also expressed in endothelial cells and platelets. NPY is elevated during stress and is a powerful vasoconstrictor, a modulator of immune functions and of neointimal hyperplasia proposed to contribute to neurogenic atherogenesis (Sobey, 2003). The elevation of this transcript during stress confirms the success of the stress regimens. Histological and serological analysis suggests that the source of NPY at three months may be endothelial. In addition, NPY has been described to have a role in inflammatory processes, and in leukocyte redistribution and adhesion (Bedoui et al., 2003; Sung et al., 1991). Fabp facilitates the uptake of fatty acids by macrophages and regulates common pathways between inflammatory response and metabolic signaling (Makowski et al., 2001; Makowski and Hotamisligil, 2004). In fact, it evokes a strong inflammatory response, with positive signals for macrophage accumulation and damage in atherosclerosis (Furuhashi et al., 2008; Furuhashi and Hotamisligil, 2008; Makowski et al., 2001). Adfp is an adipose differentiation-related protein upregulated in atherosclerotic lesions and a marker of lipid-containing cells including macrophage foam cells (Heid et al., 1998; Wang et al., 1999), where it prevents lipid efflux (Larigauderie et al., 2006; Larigauderie et al., 2004). Fabp3, which has an important role in fatty-acid transportation and is mainly produced by heart and skeletal muscle cells, has been involved in cell death as well (Cybulsky et al.). Adfp is a specific cell marker of lipid accumulation, occurring in a variety of cell types, including endothelial cells and in macrophages, where it is able to prevent lipid efflux (Heid et al., 1998; Larigauderie et al., 2004). Elevation of Fabp3 and Adfp suggests that stress can also induce lipid metabolism alterations.

The changes observed at three months suggested the potential for development of atherogenic processes during stress. Thus, the experiments were continued for a longer period of time, where some animals were utilized at different time-points to evaluate the possible formation of atheromas up to 12 months of treatment. At this time-point, stress and atherogenic diet alone elevated similarly the number of CD8 cells and macrophages, but not those of CD4 cells and neutrophils. At 3 months stress remained the only factor affecting the number of DC. Instead the combination of stress and atherogenic diet had synergistic effect on the number of CD4 cells, CD8 cells, macrophages and neutrophils. Our observations suggest that these effects may be at least in part mediated by E-selectin, whose transcript was not influenced by stress or atherogenic diet alone but increased approximately 16,000-folds when both treatments were combined. A similarly large synergistic increase was also found for the transcript of NPY, and to a lesser extent, for Adfp.

Considering the synergistic action of stress and atherogenic diet on the level of NPY and E-Selectin transcripts in aorta tissue, measurements of serum circulating levels of NPY and the soluble form of E-selectin were also carried out. Stress and high cholesterol induced only a very modest increase in circulating soluble NPY and E-selectin indicating they are not suitable serological biomarkers for the conditions tested. This discrepancy also suggests that increase in E-selectin transcripts likely reflects elevation of the membrane form of this adhesion molecule rather than the soluble form, although both typically produced by

endothelial cells. A significant source of NPY mRNA, may instead be represented by platelets found as aggregates in aortas from HCS groups.

Despite the changes observed in the number of leukocytes and adhesion molecules, histological analysis showed that the effects of stress were not more severe than those induced by atherogenic diet. Instead, stress suppressed the development of intimal foamy cells found in mice fed the atherogenic diet. Perhaps the leaner phenotype induced by the stress regimen used contributes at least in part to this difference. The accumulating classic inflammatory cells, macrophages and DCs, in stress and diet were also present in the intima, but some level of medial penetration was observed for CD68+ macrophages. Lymphocytes have been described to be associated with the adventitia (Galkina and Ley, 2007), but we have not investigated whether the risk factors affect their distribution in our model. Paradoxically, morphological changes of the aorta when the two factors were combined were mild when compared to atherogenic diet alone.

The differences in expression of molecular markers and accumulated cell types induced by stress and diet over time substantiate the complexity of this pathology and the dynamic character of the atherogenic process, even during early stages. Our results suggest that mental/psychological stress changes the inflammatory environment, altering levels and/or the type of molecules mediating the interaction between circulating leukocyte subsets and the arterial cell wall. This is in agreement with previous observations. The stress hormones can modulate endothelial phenotype and function, affecting for instance adhesion, thrombogenesis and cytokine secretion (Fantidis), causing the development of a pro-inflammatory environment.

In summary, our studies demonstrate that chronic stress can appreciably influence the local immune environment in the aorta of WT mice, and identify some of the molecules that may be responsible for this modulation. They also showed that over time the combined action of chronic stress and atherogenic diet had strong synergistic effects on the number of cells of immune origin found in the aortas but, paradoxically, not on the severity of changes to the arterial wall.

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Figure 1. Effects of Stress and High Cholesterol diet in the aorta inflammatory and molecular environment

Histograms showing the fold change of leukocytes subsets (A and C) and molecular profiling (B and D) of aortas from animals treated for 3 months (A and B) or 12 months (C and D) as indicated. Measurements were carried out by flow cytometry analysis of aorta cell suspensions or by qRT-PCR. For clarity, results were normalized to the mean value of NDC groups that arbitrarily assigned the value 1. NDC, normal diet control; NDS, normal diet stress; HCC, high cholesterol control; HCS, high cholesterol stress. Two-way ANOVA was followed by Bonferroni's post hoc test ($n=6$ animals/group in 3 months experiments and 5 animals/group in 12 months experiments). * $p<0.05$ compared to groups indicated by lines.

Figure 2. Morphological analysis

Representative grayscale pictures of paraffin embedded-aorta tissue sections stained using Hematoxylin and Eosin (H&E) from mice treated as indicated for 3 or 12 months. NDC, normal diet control; NDS, normal diet stress; HCC, high cholesterol control; HCS, high cholesterol stress. 20× magnification. Scale bar is 500um.

Figure 3. Immunohistochemical staining of NPY and E-Selectin in animals that received stress and/or high cholesterol diet for 12 months

A) Fresh aorta tissue was stained using fluorescent-labeled antibodies in a stepwise manner. 100× magnification. B) Paraffin-embedded tissue sections were treated with citrate buffer pH 6.8 for antigen retrieval, and stained using a peroxidase-based method as described. L= Lumen. Isotype control corresponds to staining using biotinylated secondary antibodies in the absence of primary antibodies, followed by Streptavidin HRP. 100× magnification. Scale bar is 100μm.

Figure 4. Macrophage distribution in the aorta wall

Three-dimensional surface plot of a z-stack image from the lesser curvature of a HCS aortic arch showing the distribution and penetration of CD68+ macrophages (green), and CD11c+ dendritic cells (orange), compared to with CD31+ endothelial cells (red). The 3D analysis was performed in ImageJ software (NIH), and indicates the presence of CD68+ cells associated with the intima (0 value on z-axis), and the penetration of those cell types towards the adventitia (200 value on z-axis).

Figure 5. Profile of aortic P-selectin transcript and presence of platelet aggregates

A) Histogram showing the fold change in the level of p-Selectin transcript measured in the aortas of mice treated for 3 or 12 months as indicated. Values are expressed in Mean $\pm$ SD of fold changes above the level of the average found in normal diet controls (NDC) groups that were assigned the arbitrary value 1. Two-way ANOVA was followed by Bonferroni's post hoc test (n=6 animals per group). *p<0.05 for comparisons indicated by lines. B) Representative H&E staining of aorta section of animal from HCS group, showing a platelet aggregate associated to the endothelial intima.

Figure 6. Histograms showing the levels of circulating E-Selectin and NPY

(A) Soluble E-Selectin and (B) NPY in the serum of mice treated as indicated. Soluble proteins were measured in the serum harvested at termination using ELISA/EIA commercial kits. Values were expressed in Mean $\pm$ SD (n=5–6 animals/group). Two-way ANOVA was followed by Bonferroni's post hoc test. *p<0.05 for comparisons indicated by lines.

Table 1

Relative expression of genes contributing to atherosclerosis in the arch of the aorta from animals that received stress and/or high cholesterol diet for 3 months or 12 months, and controls.

Genes (ddCT $\times 10^2$)	3 months treatment				12 months treatment			
	NDC	NDS	HCC	HCS	NDC	NDS	HCC	HCS
Adfp	4.3 $\pm$ 3.3	58.0 $\pm$ 7.6**	10.5 $\pm$ 3.8	25.8 $\pm$ 4.3*	2.0 $\pm$ 0.7	23.2 $\pm$ 11.5*	40.7 $\pm$ 14.5*	225.6 $\pm$ 86.4*
Fabp3	34.9 $\pm$ 10.9	531.9 $\pm$ 37.6**	34.8 $\pm$ 37.5	213.2 $\pm$ 19.9**	22.3 $\pm$ 7.9	12.4 $\pm$ 0.5	65.4 $\pm$ 18.5*	201.1 $\pm$ 41.5*
NPY	0.6 $\pm$ 0.7	4.8 $\pm$ 1.1*	0.2 $\pm$ 0.2	16.9 $\pm$ 2.6**	1.1 $\pm$ 0.6	5.4 $\pm$ 3.1	0.2 $\pm$ 0.1	3411.6 $\pm$ 241.2**
E-Selectin	3.1 $\pm$ 2.1	1309.9 $\pm$ 102.3**	10.9 $\pm$ 7.4	298.2 $\pm$ 28.9**	3.1 $\pm$ 2.1	4.8 $\pm$ 3.3	1.1 $\pm$ 0.6	16001.7 $\pm$ 1131**
L-Selectin	5.4 $\pm$ 3.3	3.4 $\pm$ 6.3	15.4 $\pm$ 14.2	13.1 $\pm$ 10.9	62.9 $\pm$ 36.2	396.8 $\pm$ 273.8	324.9 $\pm$ 222.8	221.9 $\pm$ 15.6*
P-Selectin	183.5 $\pm$ 77.1	2289.3 $\pm$ 106.4*	233.0 $\pm$ 183.7	39884.9 $\pm$ 2778*	77.8 $\pm$ 28.1	16.6 $\pm$ 11.7*	378.2 $\pm$ 134.6*	275.4 $\pm$ 18.31*
Icam-1	130.8 $\pm$ 47.7	18743.4 $\pm$ 1237*	166.3 $\pm$ 102	8680.3 $\pm$ 778.9*	262.3 $\pm$ 37.3	1329.8 $\pm$ 82.1**	869.2 $\pm$ 170.2*	683.9 $\pm$ 98.3*
Vcam-1	172.2 $\pm$ 51.1	34515.7 $\pm$ 1227**	160.8 $\pm$ 100.9	64260 $\pm$ 3917**	80.3 $\pm$ 4.8	525.6 $\pm$ 312.3*	1040.1 $\pm$ 97.5*	956.6 $\pm$ 676.3*
Vwf	62.8 $\pm$ 34.2	77.4 $\pm$ 12.4	912.7 $\pm$ 60.4**	79.9 $\pm$ 8.3	26.9 $\pm$ 1.4	20.4 $\pm$ 1.3	471.9 $\pm$ 148.7**	1169.9 $\pm$ 82.7**

Values are expressed in Mean $\pm$ SD of ddCT of 5 animals/group, calculated upon normalization using the average of GAPDH and β -actin CTs as internal controls. Primers for qRT-PCRs were purchased from SABiosciences (Frederick, MD). NDC, normal diet control; NDS, normal diet stress; HCC, high cholesterol control; HCS, high cholesterol stress. Two-way ANOVA was followed by Bonferroni's post hoc test.

* p<0.05;

** p<0.01 in comparison to NDC.